IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Toshikazu Nakamura
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DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir,

I, Kunio MATSUMOTO declare that:

I was born in Suzaka city, Nagano Prefecture, Japan, on February 5, 1959;

I am a citizen of Japan and a resident of 25-2-204, Onoharahigashi, 6-Chome, Minoo-shi, Osaka 562-0031, Japan;

I took the doctor degree on the study of "Analysis of intermolecular relationship in photosynthetic oxygen evolving complex" at Osaka University, Osaka, Japan, in 1986,

I was appointed as Assistant Professor of Department of Osaka University Medical School, Osaka, Japan in 1987;

I was appointed as Assistant Professor of Faculty of Science, Department of Biology at Kyushu University, Fukuoka, Japan in 1990;

I was appointed as Assistant Professor of Division of Biochemistry, Department of Oncology, Biomedical Research Center at Osaka University Medical School, Osaka, Japan in 1993;

I was appointed as Associate Professor of Division of Molecular Regenerative Medicine, Course of Advanced Medicine, at Osaka University Graduate School of Medicine, Osaka Japan in 1994;

I was a visiting Associate Professor of Division of Biology and Medicine at Brown University, U.S.A., from August in 1998 till July in 1999;

I reported the following papers:

1. K. Matsumoto, Y. Azuma, M. Kiyoki, H. Okumura, K. Hashimoto, and K. Yoshikawa:
Involvement of endogenously produced 1,25-dihydroxy-vitamin D3 in the growth and differentiation of human keratinocytes. ***Biochim. Biophys. Acta.***, 1092, 311-318 (1991)

2. K. Matsumoto, K. Hashimoto, K. Yoshikawa, and T. Nakamura: Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. ***Exp. Cell Res.***,196, 114-120 (1991)

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10. H. Ohmichi, U. Koshimizu, K. Matsumoto and T. Nakamura: Hepatocyte growth factor (HGF) acts as a mesenchynal-derived morphogenic factor during fetal lung development. ***Development***, 125, 1315-1324 (1998)

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12. K. Matsumoto, H. Kataoka, K. Date and T. Nakamura: Cooperative interaction between α-chain and β-chain of HGF on c-Met receptor confers ligand-induced receptor tyrosine phosphorylation and multiple biological responses. ***J. Biol. Chem.***, 273, 22913-22920 (1998)

13. K. Date, K. Matsumoto, K. Kuba, H. Shimura, M. Tanaka and T. Nakamura: Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. ***Oncogene***,17, 3045-3054 (1998)

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15. M. Tahara, K. Matsumoto, T. Nukiwa and T. Nakamura:

Hepatocyte growth factor leads to recovery from alcohol-induced fatty liver in rats. ***J. Clin. Invest.***, 103, 313-320 (1999)

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17. K. Kuba, K. Matsumoto, K. Date, H. Shimura, M. Tanaka, and T. Nakamura: HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppress tumor growth and metastasis in mice. ***Cancer Res.***, 60, 6737-6743 (2000)

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19. K. Matsumoto, H. Yoshitomi, J. Rossant, and K. Zaret: Liver organogenesis promoted by endothelial cells prior to vascular function. ***Science***, 294, 559-563 (2001)

20. D. Tomioka, N. Maehara, K. Kuba, K. Mizumoto, M. Tanaka, K. Matsumoto, and T. Nakamura: Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells by NK4 in an orthotopic mouse model. ***Cancer Res.***, 61, 7518-7524 (2001)

The experiment set out below was conducted under my supervision.

Experiment

The inhibitory effect of HGF/NK4(del 5) on the growth of human dermal microvascular endothelial cells

1. Test Method

The polypeptide of SEQ ID No: 2 (in this Declaration, referred to as HGF/NK4(del 5)) was used as a test compound.

Using human dermal microvascular endothelial cells (HMVEC-D; Clonetics) as tester vascular endothelial cells, the growth inhibitory effect of HGF/NK4(del 5) on endothelial cells was evaluated.

By use of human microvascular endothelial cells in the logarithmic phase of growth of passages 5-8, a cell suspension was prepared and a geltin-coated 24-well plate was seeded with 8000 cells per well. After 24 hours, the medium was changed to the fresh one (a 1:1 mixture of EGM (Eagle General Medium) and DMEM (Dulbecco's Modified Eagle Medium) supplied with 5% serum), and four groups of 3 ng/ml HGF, 10 ng/ml VEGF (vascular endothelial growth factor) and negative control (5% serum containing solution; None on the Drawing) were established. Then, HGF/NK4(del 5) with various concentrations of 0 to 1000 nM was added and the plate was incubated under 5% CO_2 at 37°C. After 72 hours, the cells were detached by trypsin coating and counted using a Coulter counter.

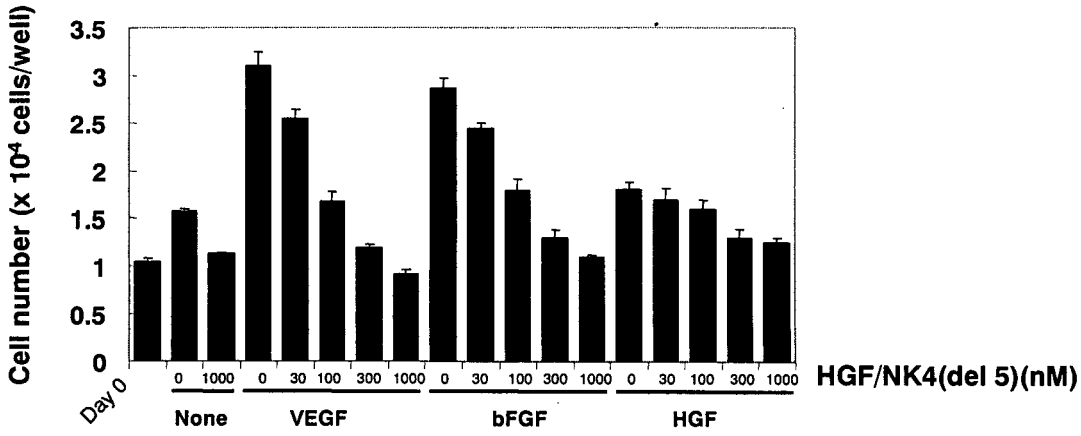
2. Test Result

The result is shown in Figure 1.

As is clear from Figure 1, the HGF/NK4(del 5) inhibits the growth of vascular endothelial cells as induced by stimulation with 10 ng/ml VEGF, 3 ng/ml bFGF, 10 ng/ml HGF and 5% serum, respectively, all concentration-dependently and siginificantly.

These results suggested that the NK4(del 5) acts in an inhibitory way not only against HGF-induced growth of vascular endothelial cells but also against the growth induced by other vascular endothelial cell growth factors such as bFGF and VEGF.

Fig. 1. Inhibitory Effect of HGF/NK4(del 5) on Growth of Human Dermal Microvascular Endothelial Cells



It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

This 30th day of August, 2006

K. Matsumoto

Kunio MATSUMOTO

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Kringle 1–4 of Hepatocyte Growth Factor Inhibits Proliferation and Migration of Human Microvascular Endothelial Cells

Keiji Kuba,*,† Kunio Matsumoto,* Kenji Ohnishi,*,[1] Takayuki Shiratsuchi,*,[1] Masao Tanaka,† and Toshikazu Nakamura*,[2]

*Division of Biochemistry, Department of Oncology, Biomedical Research Center, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan; and †Department of Surgery and Oncology, Kyushu University Graduate School of Medical Science, Maidashi, Fukuoka 812-8582, Japan

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NK4 composed of the N-terminal hairpin and subsequent four-kringle domains of Hepatocyte growth factor (HGF) is bifunctional, acting as a competitive antagonist for HGF and an angiogenesis inhibitor. In this study, we determined whether or not four-kringle domains of HGF (K1–4) have anti-angiogenic activity. For this purpose, we prepared recombinant K1–4 and NK4, using the baculovirus expression system. Although NK4 antagonized HGF-induced DNA synthesis of rat hepatocytes, cell scattering of MDCK cells and the c-Met/HGF receptor tyrosine phosphorylation in endothelial cells, K1–4 failed to antagonize HGF-induced DNA synthesis, cell scattering and the c-Met/HGF receptor tyrosine phosphorylation in endothelial cells, thus, indicating that K1–4 lacks HGF-antagonist activity. However, endothelial proliferation and migration induced by HGF was inhibited by K1–4, similar to the case seen with NK4. Furthermore, K1–4 inhibited the proliferation and migration of human dermal microvascular endothelial cells induced by vascular endothelial growth factor or by basic fibroblast growth factor. We propose that kringle 1–4 of HGF inhibits angiogenic responses in endothelial cells, independently of HGF-c-Met signaling pathways.

Key Words: **angiogenesis inhibitor; hepatocyte growth factor; HGF-antagonist; kringle domain; NK4.**

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is regulated by a balance between angiogenic and angioinhibitory molecules (1, 2). In the activated endothelium, angiogenic growth factors predominate, whereas vascular quiescence is achieved by dominance of angioinhibitory factors. Positive and negative regulation of the angiogenic switch that occurs in a time- and site-dependent manner facilitates the physiological angiogenesis involved in embryonic development, wound healing, and tissue regeneration. On the other hand, aberrant angiogenesis participates in the onset of pathology such as tumor angiogenesis and diabetic retinopathy (3, 4). Since angiogenesis is closely associated with both physiological and pathological processes, angiostatic factors as well as angiogenic factors, have potential therapeutic value for treatment of diseases seen in the clinic.

Hepatocyte growth factor (HGF) which was originally identified and cloned as mitogen for hepatocytes (5, 6), regulates malignant behavior in a variety of tumors, by inducing invasive, angiogenic, and metastatic responses (7, 8). We earlier prepared a specific antagonist for HGF, called NK4 (or HGF/NK4), by elastase-digestion of HGF (9–11). NK4 is an internal fragment of HGF and is composed of an N-terminal hairpin and four-kringle domains of the α-chain of HGF. NK4 binds to the c-Met/HGF receptor tyrosine kinase, but does not activate the c-Met receptor, thereby competitively inhibits biological responses driven by HGF-Met receptor coupling (9–11).

In our most recent study, we found that NK4 functions as an angiogenesis inhibitor and that this angiostatic activity of NK4 is probably independent on its original activity as the competitive antagonist for HGF (12). NK4 inhibits endothelial cell proliferation and migration driven by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor

Abbreviations used: bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; HMVECs, human adult dermal microvascular endothelial cells; K1–4, kringle 1–4 of HGF; S.E., standard error; VEGF, vascular endothelial growth factor.

[1] Present address: Fujii Memorial Research Institute, Otsuka Pharmaceutical Co., Ltd., 1-11-1 Karasaki, Otsu, Shiga 520-0106, Japan.

[2] To whom correspondence should be addressed. Fax: +81-6-6879-3789. E-mail: nakamura@onbich.med.osaka-u.ac.jp.

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(VEGF), as well as HGF. However, since the c-Met/HGF receptor is expressed in vascular endothelial cells and HGF is an angiogenic factor, we could not exclude the possibility that the antagonistic action of NK4 on HGF-c-Met receptor coupling participates in angioinhibitory actions of NK4. To examine this possibility, the N-terminal hairpin domain, an essential domain for binding to the c-Met receptor, was deleted from NK4. We now report that the deletion of the N-terminal hairpin domain in NK4 led to a selective loss of HGF-antagonist activity, yet the remaining part, i.e., four-kringle variant of HGF (K1–4) was angiostatic.

MATERIALS AND METHODS

Materials. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells transfected with human HGF cDNA (6, 13). Human recombinant bFGF and VEGF165 were obtained from R&D Systems (Minneapolis, MN).

Expression and purification of recombinant NK4 and K1–4. The human NK4 cDNA was amplified from a human HGF cDNA by standard polymerase chain reaction (PCR) with primers NK4-KF (5′-ACGCGTCGACCAGACCGTGCATCATGTGGGTGACCAAACTCCT-3′, forward primer for residues 1–7) and NK4-hisR (5′-CCGCTCGAGCTAGTGATGGTGATGGTGATGCGATCCTCTAGATATTACGGGATGGTCTAA-3′, reverse primer for residues 480–486 and carboxyl-terminal histidine tag), as described elsewhere (14). The amplified cDNA was ligated into Sma I site of the baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA). For construction of K1–4, two PCR products encoding HGF amino acid residues 1–66 and residues 100–486 were synthesized, using the following two sets of primers; NK4-KF and NK4-delR (5′-CACTTGACATGCTATTGAAGGGAGTATTCACTTTTTTGGTTTTTATCTTCAG-3′, reverse primer for residues 57–66 and 100–107), and NK4-delF (5′-CTGAAGATAAAAACCAAAAAAGTGAATACTCCCTTCAATAGCATGTCAAGTG-3′, forward primer for residues 57–66 and 100–107) and NK4-hisR, respectively. The two PCR products were mixed and used as a template in a final PCR with primers NK4-KF and NK4-hisR, to produce a cDNA encoding the fusion peptide.

Recombinant baculovirus generated by co-transfection of 2 μg expression plasmid and 0.5 μg Baculogold DNA (Pharmingen) was plaque-purified, propagated and used to infect 10-liter batches of Sf9 cells. The culture media containing recombinant NK4 and K1–4 were respectively dialyzed against Tris-buffered saline, pH 7.4. The NK4 and K1–4 were purified with affinity chromatography on 2 ml nickel-nitrilotriacetic acid columns (Qiagen Inc., Valencia, CA), using a gradient of 40–400 mM imidazole in 0.3 M NaCl, 0.01% Tween-80, 20 mM Tris-HCl, pH 7.4. The eluted proteins were respectively dialyzed against 20 mM citrate-NaOH buffer (pH 6.5) containing 0.5 M NaCl and 0.01% Tween-80, and concentrated with Centriprep30 (Amicon, Danvers, MA). Protein concentration was determined using micro BCA protein assay kits (Pierce, Rockford, IL) with bovine serum albumin as the standard. The level of endotoxin in the purified NK4 or K1–4 was determined to be negligible using a limulus amebocyte lysate kit from BioWhittaker.

Cell culture. Human adult dermal microvascular endothelial cells were purchased from Clonetics (San Diego, CA) and were grown in endothelial basal medium (EBM-2) supplemented with 5% fetal bovine serum (FBS) and endothelial cell growth supplements (Clonetics). Madin-Darby canine kidney (MDCK) epithelial cells, a generous gift from Dr. R. Montesano (University of Geneva) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with streptomycin, penicillin, and 10% FBS.

Cell proliferation and scattering assay. For measurement of endothelial cell proliferation, human adult dermal microvascular endothelial cells were plated at 5×10^3 cells/cm^2 onto gelatinized 24-well tissue culture plates and cultured for 24 h. The medium was replaced with 0.5 ml of EBM-2 containing 5% FBS, and cells were cultured in the absence or presence of NK4 or K1–4, 10 ng/ml HGF, 3 ng/ml bFGF, 10 ng/ml VEGF, or their combinations. After 72 h, cells were dispersed by trypsin and counted by Coulter counter. DNA synthesis of adult rat hepatocytes in primary culture was measured, as described elsewhere (6, 14). For cell scattering assay, MDCK cells were seeded on a 24-well plate at the density of 5×10^3 cells/well in DMEM supplemented with 5% FBS, with or without test samples. The cells were cultured for 20 h, stained with hematoxylin, and photographed. All these experiments were independently performed three times and similar results were obtained in each experiment.

Endothelial cell migration assay. Migration of human adult dermal microvascular endothelial cells was evaluated using a modified Boyden chamber assay, as described (15, 16). The cells were serum-starved in EBM-2 medium for 12 h and plated at 12×10^4 cells/cm^2 onto the polycarbonate filter with 5-μm pores (Costar, Cambridge, MA) coated with 13.4 μg/ml fibronectin (Orgagnon Teknika Corp., West Chester, PA). Test samples were added to the medium in the outer cup, and the cells were cultured for 5 h. The number of the cells which migrated to the undersurface of the filter was quantified by counting the cells in randomly selected five microscopic fields (×200) in each well.

Immunoprecipitation and Western Blot. Tyrosine phosphorylation of c-Met was analyzed, as described elsewhere (10). Briefly, human adult dermal microvascular endothelial cells were grown on 100-mm plates and serum starved overnight, prior to treatment for 10 min with NK4 or K1–4 (300 nM each), in the presence or absence of HGF (10 ng/ml). Cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM Na_3VO_4, 100 mM NaF, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin for 1 h at 4°C. Equivalent amounts of protein were incubated overnight with a monoclonal antibody against phosphotyrosine (PY99, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation for 2 h with protein G-Sepharose. The immunoprecipitates were washed four times with lysis buffer and subjected to SDS-PAGE, under reducing conditions, using 6% polyacrylamide gel. The proteins were electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA) and probed with anti-c-Met antibodies (C-12) (Santa Cruz Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) system (Amersham, Little Chalfont, UK).

Data analysis. Data represent the mean ± SE. Statistical analyses were performed with unpaired Student's t test (two-tailed). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Purification and HGF-antagonist activity of recombinant NK4 and K1–4. To examine the biological activities of NK4 and K1–4, recombinant NK4 and K1–4 were expressed in Sf9 insect cells. For efficient purification, NK4 and K1–4 C-terminally contained histidine tags (Fig. 1A). When affinity-purified NK4 and K1–4 were subjected to SDS-PAGE and followed by protein staining, NK4 and K1–4 appeared as a single band with Mr of 67 kD and 63 kD, respectively (Fig. 1B). Western blotting with mouse anti-HGF monoclonal antibody also showed the same single band for each protein (not shown). The predicted Mr of NK4 and of

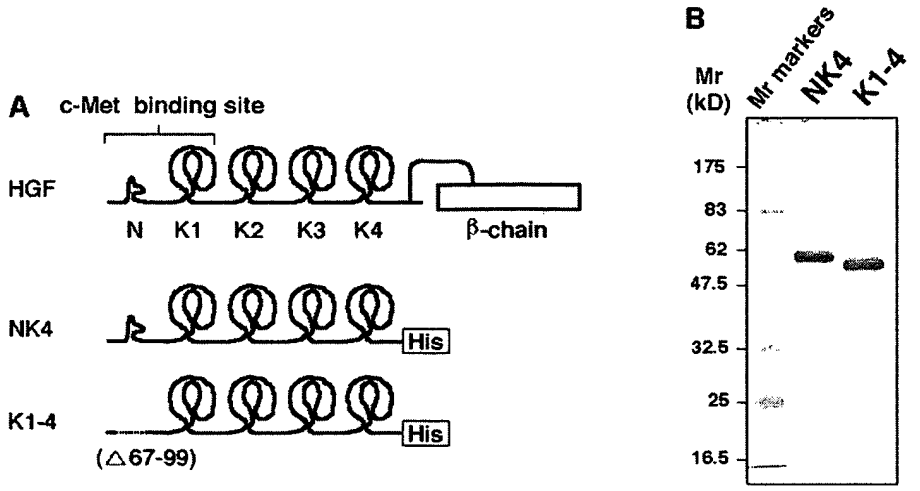

FIG. 1. (A) Schematic representation of HGF and recombinant NK4 and K1–4. Both hairpin (N) and kringle 1 (K1) in HGF are domains essential for binding to the c-Met receptor. K1–4 was generated by deletion of the hairpin domain (amino acids 67–99) from NK4. NK4 and K1–4 C-terminally contained histidine-tag (His). (B) Characterization of purified recombinant NK4 and K1–4. NK4 and K1–4 (2 μg each) were subjected to SDS-PAGE under reducing conditions, using 10% polyacrylamide gel, and proteins were stained with Coomassie brilliant blue.

K1–4 was 54 kD and 50 kD, respectively. The difference between the predicted Mr and those of purified proteins under SDS-PAGE may be due to glycosylation, since there are three glycosylation sites in kringle domains (6, 13, 17, 18). Densitometric analysis of stained polyacrylamide gel indicated that purity of NK4 and K1–4 was 94% and 96%, respectively, and we could purify 300–500 μg NK4 and 200–400 μg K1–4 from one liter of culture medium. Thus, the highly purified materials were used in the following experiments.

To determine if deletion from NK4 of N-terminal hairpin domain, an essential domain responsible for c-Met receptor binding would affect its antagonistic activity for HGF, we first examined effects of NK4 and K1–4 on HGF-induced DNA synthesis of rat hepatocytes in primary culture (Fig. 2A). HGF (0.06 nM = 5.5 ng/ml) stimulated DNA synthesis of rat hepatocytes to

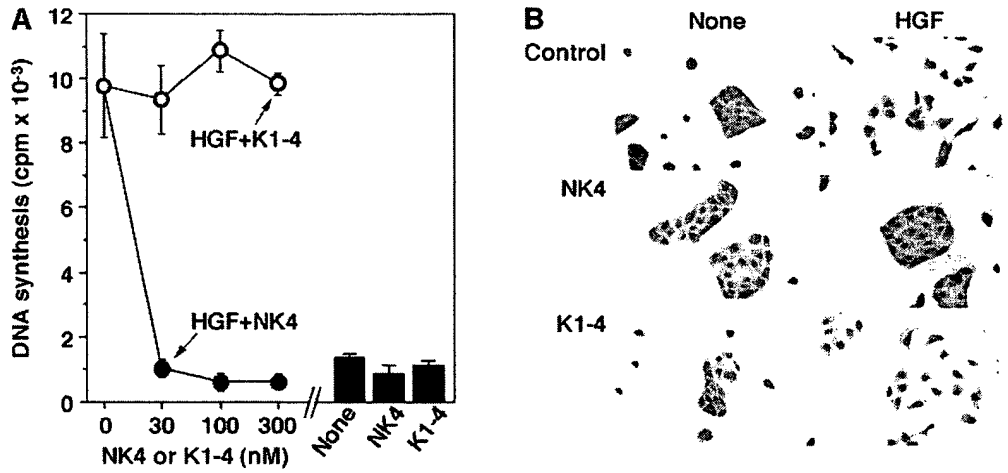

FIG. 2. Effects of NK4 and K1–4 on the mitogenic and motogenic activities of HGF. (A) Effects of NK4 and K1–4 on HGF-induced DNA synthesis of rat hepatocytes in primary culture. The cells were cultured in the absence or presence of varying concentrations of NK4 or K1–4, 0.06 nM HGF, or their combinations for 20 h, and pulse-labeled with [^{3}H]-thymidine for 6 h. The cells were also cultured in the presence of 100 nM NK4 or K1–4 alone. Each value represents the mean ± SE of triplicate measurements. (B) Effects of NK4 and K1–4 on HGF-induced cell scattering in MDCK renal epithelial cells. The cells were cultured in the absence or presence of 100 nM NK4, 100 nM K1–4, 0.11 nM HGF, or their combinations for 20 h.

a 8-fold higher level over controls, whereas neither NK4 nor K1–4 alone had stimulatory effect on DNA synthesis (Fig. 2A). When NK4 (30–300 nM) was added to cultures in the presence of HGF, NK4 abrogated DNA synthesis of hepatocytes induced by HGF. On the other hand, K1–4 (30–300 nM) had no inhibitory effect on HGF-induced DNA synthesis of hepatocytes.

We next examined effects of NK4 and K1–4 on HGF-induced cell scattering in MDCK renal epithelial cells (Fig. 2B). Although MDCK cells tightly associated colonies in control culture wihout HGF, the addition of 0.11 nM (10 ng/ml) HGF induced scattering of MDCK cells, indicating that HGF enhanced dissociation and locomotion of MDCK cells (Fig. 2B). Consistent with our previous finding (9), when 100 nM NK4 was added in the presence of HGF, NK4 abolished cell scattering of the cells, thus, indicating that NK4 antagonized the biological activity of HGF to induce cell scattering. In contrast, 100 nM K1–4 failed to inhibit HGF-induced cell scattering. Neither NK4 nor K1–4 alone stimulated scattering of MDCK cells. These results indicate that recombinant NK4 retained antagonistic activity for HGF, whereas K1–4 lost HGF-antagonist activity due to deletion of N-terminal hairpin domain in NK4.

Effects of NK4 and K1–4 on HGF-induced c-Met phosphorylation, proliferation, and migration in endothelial cells. Since HGF induces angiogenic responses through activation of the c-Met/HGF receptor in vascular endothelial cells, we examined the effects of NK4 and K1–4 on HGF-stimulated tyrosine phosphorylation of the c-Met receptor in human dermal microvascular endothelial cells in culture. The cells were serum-starved and c-Met receptor tyrosine phosphorylation upon HGF-stimulus was analyzed by immunoprecipitation and subsequent Western blotting. In quiescent cells, c-Met receptor was only faintly tyrosine-phosphorylated (Fig. 3A). The addition of 0.11 nM HGF strongly induced the tyrosine phosphorylation of c-Met receptor, whereas NK4 almost completely blocked HGF-induced c-Met tyrosine phosphorylation. In contrast, K1–4 did not block HGF-induced tyrosine phosphorylation of the c-Met receptor. Neither NK4 nor K1–4 alone induced tyrosine phosphorylation of c-Met in endothelial cells. These results indicate that HGF activated c-Met receptor and that NK4 but not K1–4 antagonized HGF-induced c-Met receptor activation in endothelial cells.

Based on the above results, we next examined effects of NK4 and K1–4 on proliferation and migration of human dermal microvascular endothelial cells. During a 3-day culture, 0.11 nM HGF stimulated cell proliferation to a 2-fold higher level over control (without growth factor), whereas 300 nM NK4 inhibited endothelial cell proliferation to the level seen in control cultures (Fig. 3B). Importantly, although K1–4 had no HGF-antagonist activity, 300 nM K1–4 significantly inhibited the endothelial cell proliferation stimulated by HGF. The inhibitory effect of NK4 on HGF-induced endothelial proliferation was more potent than that of K1–4, possibly owing to the additive effects of HGF-antagonist and angiostatic activities. In modified Boyden chamber assays, 0.11 nM HGF stimulated endothelial migration to a 2-fold higher level over controls, while both K1–4 and NK4 potently suppressed HGF-induced migration of endothelial cells (Fig. 3C). Thus, K1–4 inhibits HGF-induced proliferation and migration of endothelial cells, even though K1–4 is devoid of the potential to antagonize HGF-Met coupling.

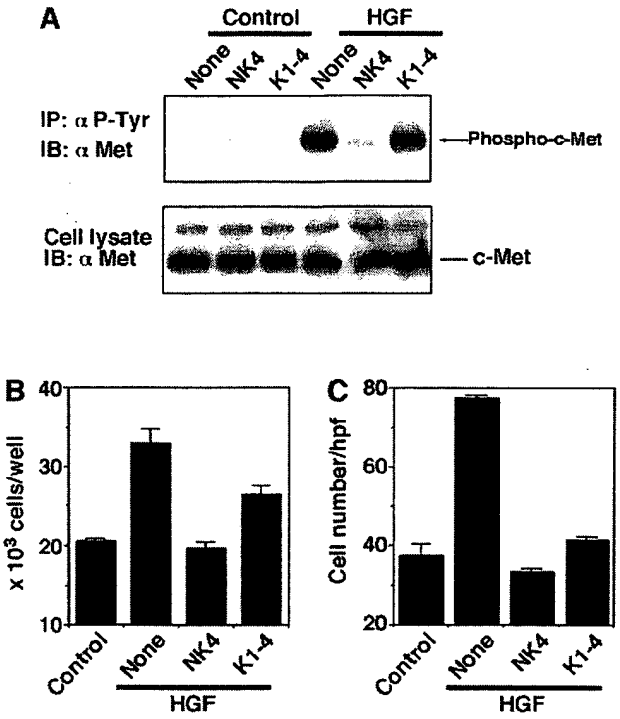

FIG. 3. Effects of NK4 and K1–4 on HGF-induced c-Met receptor tyrosine phosphorylation, proliferation, and migration in human dermal microvascular endothelial cells (HMVECs). (A) Effects of NK4 and K1–4 on HGF-induced c-Met receptor tyrosine phosphorylation. Serum-starved HMVECs were treated with 300 nM NK4 or K1–4 alone or combination with 0.11 nM HGF for 10 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (αP-Tyr) and blotted with anti-c-Met antibody (αMet). (B) Inhibition of HMVEC proliferation by NK4 or K1–4. The cells were cultured in the absence or presence of 0.11 nM HGF, without or with NK4 or K1–4 (300 nM each) for 72 h, and the number of cells was measured. (C) Inhibition of HMVEC migration by NK4 or K1–4. The cells were cultured for 5 h on a fibronectin-coated filter membrane in the absence or presence of 0.11 nM HGF, with or without NK4 or K1–4 (300 nM each). The number of cells which migrated through the membrane was counted. Each value represents the mean ± SE of triplicate measurements.

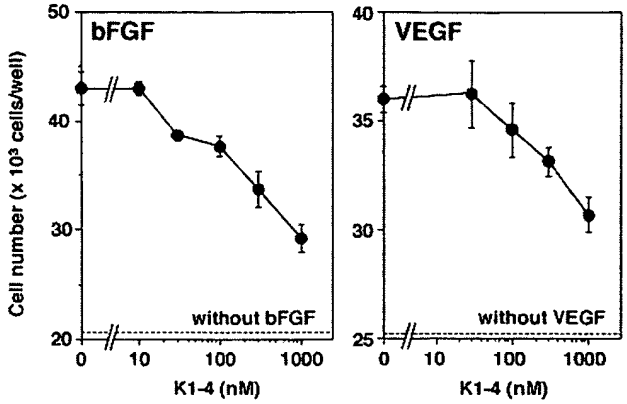

FIG. 4. Inhibitory effect of K1–4 on bFGF- and VEGF-induced proliferation of human dermal microvascular endothelial cells. The cells were cultured in the absence or presence of 0.17 nM bFGF or 0.3 nM VEGF, with varying concentrations of K1–4 for 72 h, and the number of cells was measured. Each value represents the mean ± SE of triplicate measurements.

Inhibitory effect of K1–4 on proliferation and migration of endothelial cells induced by bFGF and VEGF. To further determine K1–4 actually functions as an angiogenesis inhibitor, we tested the effect of K1–4 on bFGF- and VEGF-induced proliferation of endothelial cells. K1–4 was added to cultures of human dermal microvascular endothelial cells in the absence or presence of 0.17 nM (3 ng/ml) bFGF or 0.3 nM (10 ng/ml) VEGF. bFGF stimulated cell proliferation to a 2.0-fold higher level over control, whereas K1–4 inhibited bFGF-induced proliferation of endothelial cells, in a dose-dependent fashion, with an IC50 of approximately 300 nM (Fig. 4). Similarly, although VEGF stimulated endothelial cell proliferation, VEGF-induced endothelial proliferation was also significantly inhibited by K1–4. The inhibitory effect of K1–4 on bFGF- or VEGF-induced endothelial proliferation was the same as NK4 in its potency (not shown). It should be emphasized that K1–4 did not inhibit the proliferation of non-endothelial cells, including human dermal fibroblasts, Lewis lung carcinoma cells, MDCK cells, and rat hepatocytes (Fig. 2A and not shown). Thus, K1–4 specifically inhibited the proliferation of endothelial cells, but had no effects on the proliferation of non-endothelial cells.

We next examined the effects of K1–4 on endothelial migration. When 0.55 nM (10 ng/ml) bFGF and 0.03 nM (1 ng/ml) VEGF were respectively added to Boyden chamber assays of endothelial cells, the migration of cells was stimulated to 1.3-fold and 2.0-fold higher levels over control by bFGF and VEGF, respectively (Fig. 5). K1–4 dose-dependently inhibited bFGF- or VEGF-induced migration of endothelial cells. IC50 in inhibitory effect of K1–4 on bFGF- and VEGF-induced endothelial cell migration was approximately 30 nM. K1–4 exhibited inhibitory effect on endothelial migration at lower concentrations than those seen on endothelial proliferation.

DISCUSSION

Previous studies on the structure-function relationship in HGF indicated that N-terminal hairpin and the first kringle domain of HGF are responsible for specific binding of HGF to the c-Met receptor (19, 20), and respective deletion of these domains from the HGF molecule resulted in loss of biological activities mediated through c-Met (14, 21, 22). Consistent with these notions, deletion of the N-terminal hairpin domain from the NK4 molecule resulted in loss of its antagonistic activity for HGF-c-Met coupling. NK4 but not K1–4 abolished cell proliferation, cell scattering and c-Met receptor tyrosine phosphorylation triggered by HGF. We now have the first evidence that K1–4, which has no antagonistic action on HGF, retained inhibitory effects on endothelial proliferation and migration mediated by bFGF, VEGF, and HGF, in a similar manner to NK4. Therefore, our results indicate that kringle 1–4 of HGF has angioinhibitory action and that the previously-noted angioinhibitory action of NK4 may be mediated through four-kringle domains.

Since antagonistic effects of NK4 on HGF are not involved in its angioinhibitory action, mechanisms by which NK4 and K1–4 inhibit angiogenic responses in endothelial cells remained to be addressed. The most likely mechanism is that NK4 and K1–4 may exert angiostatic signals through putative binding molecules on endothelial cells. In this context, it is noteworthy that kringle domains in some kringle-containing molecules have angioinhibitory actions, including angiostatin (23), the fifth kringle domain of plasminogen

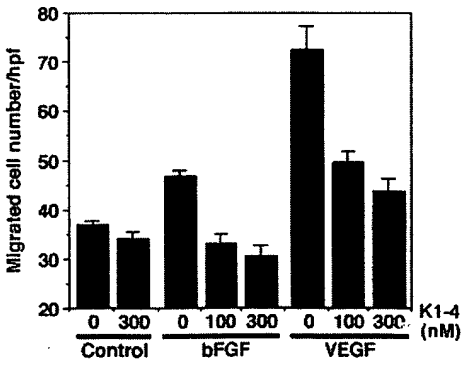

FIG. 5. Inhibitory effect of K1–4 on migration of human dermal microvascular endothelial cells induced by bFGF or VEGF. The cells were cultured on fibronectin-coated filter membrane in the absence or presence of 0.55 nM bFGF and 0.03 nM VEGF, with or without K1–4 for 5 h. The number of cells which migrated through the membrane was counted. Each value represents the mean ± SE of triplicate measurements.

(24), and the kringle-2 of prothrombin (25). The structural motif conserved in some kringle domains may possibly participate in inhibiting angiogenic responses. Moser *et al.* reported that binding of angiostatin to ATP-synthase on the plasma membrane is likely to be involved in angiostatin-induced endothelial inhibition (26). However, it is equally possible that NK4 and K1–4 inhibit angiogenesis through a mechanism distinct from angiostatin and other kringle domains, since NK4 inhibits DNA synthesis and induces cell-cycle arrest (our unpublished data), whereas angiostatin increases endothelial apoptosis without inhibiting DNA synthesis (27, 28).

The therapeutic potential of angiogenesis inhibitors have been proposed for treatment of angiogenic diseases such as malignant tumors and diabetic retinopathy wherein pathological angiogenesis could be triggered either by an up-regulation of angiogenic factors or by a down-regulation of endogenous angiostatic factors (2). Angiogenesis inhibitors have been shown to inhibit tumor growth and metastasis (23, 29–31), and some angiogenesis inhibitors are under clinical trials for cancer treatment (23, 31, 32). We found that NK4 inhibited tumor angiogenesis, invasion, and metastasis of distinct types of malignant tumors in mice (10, 12). Bifunctional characteristics of NK4 as an HGF-antagonist and angiogenesis inhibitor implicates that the therapeutic potential of NK4 may possibly exceed angiogenesis inhibitors in treating malignant tumors in which HGF-c-Met receptor system affects their malignancy.

The elucidation of angioinhibitory mechanisms of NK4 and K1–4 may lead to further understanding of molecular mechanisms involved in vascular quiescence. For this purpose, we hope to identify the putative binding partner of K1–4, if it is expressed in an endothelial specific manner.

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